Organs-on-a-Chip 2 (2020) 100003

Contents lists available at ScienceDirect

Organs-on-a-Chip

journal homepage: www.journals.elsevier.com/organs-on-a-chip

A practical guide to microfabrication and patterning of hydrogels for biomimetic cell culture scaffolds

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ARTICLE INFO

Keywords:
Microfabrication
Hydrogels
3D cell cultures
Organs-on-chip
Microfluidics

ABSTRACT

This review article describes microfabrication techniques to define chemical, mechanical and structural patterns in hydrogels and how these can be used to prepare *in vivo* like, i.e. biomimetic, cell culture scaffolds. Hydrogels are attractive materials for 3D cell cultures as they provide ideal culture conditions and they are becoming more prominently used. Single material gels without any modifications do however have their limitation in use and much can be gained by in improving the *in vivo* resemblance of simple hydrogel cell culture scaffolds. This review article discusses the most commonly used cross-linking strategies used for hydrogel-based culture scaffolds and gives a brief introduction to microfabrication methods that can be used to define chemical, mechanical and structural patterns in hydrogels with micrometre resolution. The review article also describes a selection of literature references using these microfabrication techniques to prepare organ and disease models with controlled cell adhesion, proliferation and migration. It is intended to serve as an introduction to microfabrication of hydrogels and an inspiration for novel interdisciplinary research projects.

Introduction

Cell cultures are widely used within biological and medical research as simplified model systems to study complex aspects of human biology. A major motivation for these *in vitro* models is to make the research more time efficient and economically feasible and hence they are typically used for early stage screening assays. A weakness is, however, that the cells often gradually lose their phenotype due to the simplified composition of the current culture platforms used to establish the models. In tissues and organs, cell function is dictated by biochemical and biophysical cues from the microenvironment (Barthes et al., 2014) and these are not included when the cells are cultured on two-dimensional (2D) tissue culture plastics. To address this, biomaterials, and especially hydrogels, have become widely used as a substitute for the *in vivo* extracellular matrix (O'Brien, 2011) in many *in vitro* models. Hydrogels are three-dimensional (3D) networks of hydrophilic polymers that can comprise up to 99% of water. Hydrogels have proven ideal as the base for *in vitro* cell cultures, both as 2D and 3D scaffolds (Wang et al., 2013a; Zhang et al., 2016a) due to the combination of their high porosity that allows for nutrient and gas transport and their tuneable chemical and mechanical properties (Ghaemmaghami et al., 2012).

2D biomaterial-based models (*i.e.* when the cells are cultured on the surface of a hydrogel) recapitulate more of the natural cell environment compared with 2D cultures on plastic surfaces. This is especially true for tissues such as epithelial barriers. To better emulate the microenvironment of most bodily tissues and organs a 3D microenvironment is however needed where the cells are encapsulated in the biomaterial. Additionally, an ideal *in vitro* model would also allow for spatial and temporal control of the chemical and mechanical properties of the biomaterial (Lutolf et al., 2009) as these combined control the cell fate through affecting cell morphogenesis, polarization, migration, proliferation, differentiation, and survival (Kaivosoja et al., 2012), Fig. 1. For this purpose, both naturally derived and synthetic materials have been explored. Naturally derived materials are advantageous because of their inherent biological properties, promoting cell adhesion and matrix remodelling and degradation. Synthetic materials, on the other hand, can offer greater control over the material properties and the subsequent biological responses (Lutolf and Hubbell, 2005). Further, natural polymers tend to have more inconsistent properties due to batch-to-batch

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https://doi.org/10.1016/j.ooc.2020.100003
Received 24 January 2020; Received in revised form 13 March 2020; Accepted 30 March 2020
Available online 9 April 2020

List of abbreviations

2PP	two-photon polymerisation
3T3	3-day transfer, inoculum 3 × 105 cells (a cell line)
μCP	micro contact printing
CD44	cluster of differentiation 44 (a cell-surface glycoprotein)
DMD	digital micro-mirror devices
DNA	deoxyribonucleic acid
ECM	extra cellular matrix
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
HA	hyaluronic acid
HeLa cells	Henrietta Lacks cells (a cell line)
HL-60	human leukemia cells (a cell line)
HepG2	human liver derived cells (a cell line)
hMSC	human mesenchymal stromal cells
HUVEC	human umbilical vein endothelial cells
GelMA	gelatin methacryloyl
IR	infra red
LAP	lithium phenyl-2,4,6-trimethylbenzoylphosphinate
LED	light emitting diode
PDMS	poly(dimethylsiloxane)
PEG	poly(ethylene glycol)
PEG-DA	poly(ethylene glycol) diacrylate
PS	poly(styrene)
RGD	ACRL-PEG-RGDS (a peptide sequence)
SFL	stop flow lithography
SPAAC	Strain-promoted azide-alkyne cycloaddition
UV	ultra violet

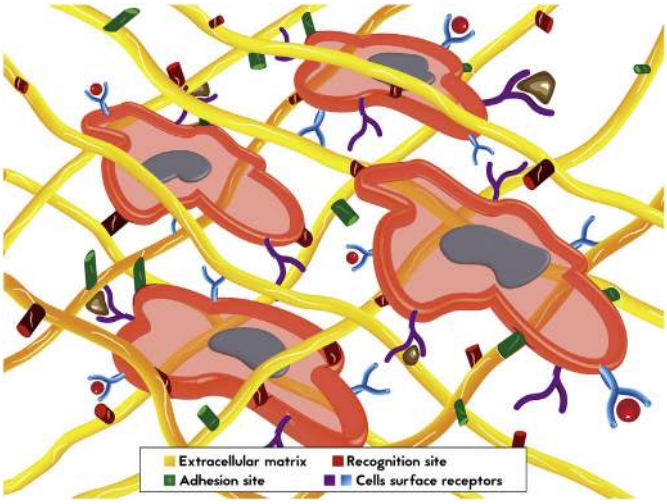

Fig. 1. Cells interact with their surrounding environment, the extracellular matrix, via mechanical and chemical cues.

variations from the extraction process, which is not the case for synthetic polymers.

The reports so far on patterned hydrogel cell culture scaffolds are limited and to date it is mostly chemical modifications of the biomaterial that have been explored. Further reports have studied cell interactions with the extracellular matrix (ECM) via soluble biochemical signals, such as cytokines and growth factors (Mieszawska and Kaplan, 2010). Others have explored how cells sense mechanical forces such as stress, strain, rigidity, topology, and adhesiveness through cues from the ECM that are transferred by ligands to the cell surface receptors (Chen et al., 2017). Due to the limited reports in literature so far, there is a window of opportunity to develop more *in vivo*–like environments in the *in vitro* models by tailoring both the chemical, mechanical and structural properties of the biomaterials used. Specifically, tailoring these properties on the same length scale as the cultured cells (typically 10–150 μm) has great potential to improve current *in vitro* models. This calls for more sophisticated fabrication methods in the biomaterials area.

Microfabrication techniques were first developed to realise integrated circuits comprising miniaturised transistors and other electronic components that enabled the digital revolution of our society. More than 25 years ago, the first report came out where microfabrication techniques were used to define chemical patterns on a surface to control cell adhesion and function (Singhvi et al., 1994). Even ten years before that, a similar study had been performed evaluating the effect of microtopography on neuronal outgrowth (Clark et al., 1987). By the end of the 1990's more studies were performed (Chen et al., 1997; Bhatia et al., 1998; Dewez et al., 1998; Zhang et al., 1999; Britland et al., 1992) and it was clear that the ability to control cell adhesion, proliferation and cell fate could be an extremely powerful tool for biological research. In the early works however, patterns were only obtained on inert and biologically non-relevant materials such as poly (dimethylsiloxane) (PDMS), glass or culture plastics and it was not until the beginning of 2000′ies that techniques to pattern biomaterials and hydrogels, either chemically (Wang et al., 2002) or mechanically (Wong et al., 2003) were developed.

With this review article, we have the ambition to contribute to an increased awareness of both simple and advanced methods to control and adjust the chemical, mechanical and structural properties of biomaterials with micrometre-sized resolution. There are many different types of biomaterials that can be used for microfabricated cell culture scaffolds and in this review article we focus on the use of hydrogels. The review article will first describe some different cross-linking strategies that can be used to prepare micropatterned hydrogel scaffolds and after that, the basic principles of different microfabrication techniques, such as UV lithography, moulding, microcontact printing (μCP) and two-phase microfluidics, will be introduced. The remainder of the review focuses on describing examples where these techniques have been used to define well-controlled cell culture microenvironments and the extra benefits gained by using them. It is the ambition of the review article to show a large range of examples to broaden the views of the readers and contribute to an even larger degree of cross-disciplinary research efforts to provide biomimetic *in vitro* models for more reliable biological and medical research. A note should be made that not all available fabrication techniques are included in the review. For example additive manufacturing, often referred to as 3D printing or bio-printing, micromilling and electrospinning are example of techniques that are omitted from the review article as these fabrication techniques do not originate from the microelectronics field which all other techniques do. The interested reader is referred to other detailed reviews such as the ones by Murphy and Atala or Ma et al. (Murphy and Atala, 2014; Ma et al., 2018) discussing additive manufacturing or the work by Pasman et al. addressing specifically electrospun organs-on-chip membranes (Pasman et al., 2018).

Hydrogel cross-linking strategies

In order to create stable cell culture scaffolds the hydrogel polymer chains need to be cross-linked to prevent degradation. The selection of cross-linking strategy is determined by the application of the biomaterial. If cells shall be encapsulated in the hydrogel for 3D cultures, the cross-linking chemistry must not be toxic to the cells. However, harsher approaches may be employed for 2D scaffolds (Sarker et al., 2018; Zhang et al., 2010) It should be noted that the methods described below are not an extensive list but only covers the approaches used in the publications

later referenced in the Section "Applications of microfabricated hydrogels".

It is important to remember that although the hydrogels used as scaffolds are cross-linked, they are still susceptible to swelling. Hydrogel swelling or shrinking depends on the chosen hydrogel material, on the cross-linking density (polymer molecular weight and degree of functionalisation) and on the osmolarity of the solution the hydrogel is incubated in. A hydrogel that is cross-linked in one solution might swell or shrink significantly over time when placed in another solution. In general, more densely cross-linked materials swell less compared to more loosely cross-linked hydrogels and the interested reader is referred to a review specialized on this topic by Bajpai et al. (Bajpai, 2001) The swelling/shrinking of the hydrogel will change its shape over time and must be taken into consideration already during the design phase of the biomimetic cell culture scaffold.

Another property of the hydrogel that is determined during the cross-linking step is the degree of porosity and pore size that later determines the ability of the scaffold to provide adequate nutrient and waste exchange for the encapsulated cells. Hydrogels are classified according to their pore size as either non-porous (10–100 Å pores), microporous (100–1000 Å pores) or macroporous (0.1–1 μm pores). Higher porosity, leading to improved diffusion in the matrix, may be tailored by i) increasing the distance between the cross-linking points i.e. the molecular weight of the used polymer or ii) increase the number of cross-linking points within the polymer chain by using multifunctional (also known as star polymers) or polymers with a higher degree of functionalisation, Fig. 2. A porosity allowing for good diffusion is beneficial for performing cell culture inside the hydrogel matrices but it may be also result in false signals regarding drug permeability in barrier models due to matrix absorption of the drug molecules.

It is important to remember that changes in the hydrogel porosity almost always also affect the mechanical strength of the gel, so that a more porous biomaterial simultaneously shows a lower Young's modulus and becomes more susceptible to degradation. During the fabrication, the hydrogel porosity can further be modified by using porogens, freeze-drying or gas-foaming and we refer the interested reader to other review articles on this topic (Annabi et al., 2010; De France et al., 2018). In some applications however, degradability of the hydrogel scaffold during cell culture is desired to enable matrix remodelling. Most natural polymers can be enzymatically degraded by mammalian cells but hydrogels that lack natural cleavage sites can be modified with functional groups that can be enzymatically degraded (Lutolf et al., 2003) or activated via light (Kloxin et al., 2009).

Table 1 lists a summary of the advantages and disadvantages of the different hydrogels that are covered in this review article.

Reversible cross-linking

Attraction of differently charged functional groups result in hydrogels that are reversibly cross-linked via strong electrostatic interactions between the ions. Collagen is the most common ECM component *in vivo* and popular for hydrogel synthesis as it provides charged functional groups for cross-linking as well as ligands for cell adhesion (Gasperini et al., 2014). Collagen and its degradation product gelatine can be readily physically cross-linked by changing pH and temperature to form a so-called thermogel (Hsiao et al., 2015), Fig. 3. Both collagen and gelatine are biocompatible, biodegradable and show low immunogenicity. These gels are therefore highly suitable for soft lithography or moulding, as shown by Wong et al. (2008). Matrigel® (also known as GelTrex) is another common hydrogel that resembles the ECM, which is reversibly cross-linked and consists of a mixture of different structural proteins and growth factors (Miller et al., 2012). Another hydrogel that is cross-linked via ionic interactions is alginate, a negatively charged polysaccharide extracted from marine algae. Divalent cations like Ca^{2+} or Ba^{2+} initiate the gelation of alginate by diffusion-controlled ionic cross-linking, Fig. 3 (Sun and Tan, 2013). Alginate is the most commonly used hydrogel for fibre production in microfluidics (Shin et al., 2007). The advantages of ionic cross-linking are that no chemical modifications of the polymer building blocks are needed and the reactions are generally highly biocompatible. The technique is also very simple, which is a major advantage when one considers large-scale production of *in vitro* models. The resulting hydrogels do however have limitations when it comes to serving as scaffolds for long-term cell cultures since thermogels made from gelatine will dissolve when heated above 40 °C and alginate hydrogels can dissolve within days due to Ca^{2+} loss (Henke et al., 2016). Further disadvantages of this cross-linking approach in relation to microstructured culture scaffolds is the fact that the reaction is diffusion dependent and thus result in an inhomogeneous cross-linking density in thicker layers. Moreover, it is difficult to achieve changes in temperature and pH locally (on the scale of tens of μm's) leading to insufficient spatial resolution using this cross-linking scheme. Although reversibly cross-linked hydrogels are less suitable as culture scaffolds themselves, they do find use in sacrificial moulding. Here, ionically cross-linked hydrogels can be used to define the final structures and removed after a heat treatment once the hydrogel scaffold has been properly cross-linked (Kolesky et al., 2016; Valentin et al., 2017).

Covalent radical cross-linking

To obtain more permanent hydrogels, the formation of covalent bonds between the polymer chains is necessary. A broadly applied strategy is to attach functional groups with double bonds that can undergo radical reactions, triggered by an external stimuli such as light or heat. Again, precise temperatures are typically difficult to define locally in a hydrogel so photo-initiated reactions are easier to use. The most prominent example of employed functional groups are acrylates, methacrylates or acrylamides. Hyaluronic acid (HA) (Wade et al., 2015; Khetan and Burdick, 2010; Skardal et al., 2015; Márquez-Posadas et al., 2013), chitosan (Kufelt et al., 2015), dextran (Henke et al., 2016), gelatine, and poly (ethylene glycol) (PEG) (Miller et al., 2012; Panda et al., 2008; Cuchiara et al., 2010; Liu and Bhatia, 2002; Hati et al., 2018; Urrios et al., 2016; Lee et al., 2009a, 2011, 2014; Liu Tsang et al., 2007) are all examples of polymers that have been functionalised with acrylates for cross-linking purposes.

In addition to the external stimuli, a chemical initiator also needs to be mixed into the polymer solution to achieve cross-linking, Fig. 3. The two most commonly used photo-initiators are Irgacure 2959 and lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) (Fairbanks et al.,

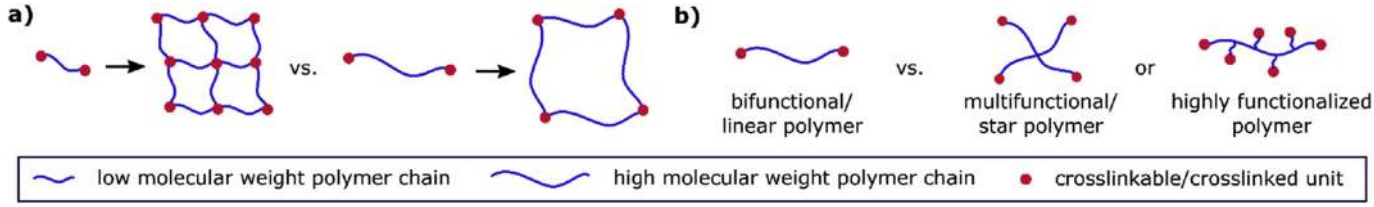

Fig. 2. Ways to affect hydrogel porosity by a) changing the molecular weight of the employed polymer and thereby increasing the distance between the crosslinking points or b) using multifunctional polymers or polymers with a high degree of functionalisation instead of a bifunctional polymer. The porosity is critical for the provision of nutrients and to allow for cell migration within the hydrogel (if desired).

Table 1
Advantages and disadvantages of polymer precursors used for hydrogel synthesis described in this review article. There are many other types of hydrogel materials available, here we only list those used in the applications addressed in the later sections. For more detials on hydrogel chemistry, the interested reader is referred to other review articles on this topic (Spicer, 2020; Hunt et al., 2014).

Polymer	Advantages	Disadvantages	Reaction chemistries and references
SYNTHETIC POLYMERS			
Poly (ethylene glycol) (PEG) PEG-diacrylate PEG-dimethacrylate PEG-azide	• Biologically inert • Dissolvable in different solvents which enables easy functionalisation reactions • Inexpensive • No batch to batch variation • Strong mechanical properties	• No promotion of cell attachment • Not enzymatically degradable	Photo-initiated radical cross-linking (Miller et al., 2012; Panda et al., 2008; Cuchiara et al., 2010; Liu and Bhatia, 2002; Hati et al., 2018; Urrios et al., 2016; Lee et al., 2009a; Liu Tsang et al., 2007; Li et al., 2011, 2014; Warner et al., 2016; Chan et al., 2010; Accardo et al., 2018; Du et al., 2008; Pregibon et al., 2006; Kizilel et al., 2004; Hahn et al., 2006; Miri et al., 2018; Burtch et al., 2018; Fu et al., 2014; Hammoudi et al., 2010; Koh et al., 2003; Larsen et al., 2014) Redox-initiated radical cross-linking (Kloxin et al., 2009; Tibbitt et al., 2010) Strain-promoted azide-alkyne cycloaddition (SPAAC) cross-linking (DeForest et al., 2009; DeForest and Anseth, 2011; Caldwell et al., 2017) Thiol-ene click cross-linking (Skardal et al., 2015; Li et al., 2018; Gobaa et al., 2011)
Poly (acrylamide)	• Biologically inert • Inexpensive • No batch to batch variation	• No promotion of cell attachment • Monomers are cytotoxic • Not enzymatically degradable	Photo-initiated radical cross-linking (Hynd et al., 2007a, 2007b) Redox-initiated radical cross-linking (Abdeen et al., 2014; Benedetto et al., 2005; DePorter et al., 2012; Lee et al., 2013)
NATURAL POLYMERS			
Polysaccarides			
Hyaluronic acid (HA) HA-acrylate HA-methacrylate HA-thiol HA- norbornene	• Enzymatically degradable • Biocompatible • Interacts with CD44 receptors	• Viscous solution • No promotion of cell attachment	Photo-initiated radical cross-linking (Khetan and Burdick, 2010; Márquez-Posadas et al., 2013; Qin et al., 2014) Thio-ene click cross-linking (Wade et al., 2015; Skardal et al., 2015) Carbodiimide cross-linking (Goubko et al., 2010, 2014)
Agarose	• Biologically inert • Dissolves upon heating and gels upon cooling	• Not biodegradable • No promotion of cell attachment • Hydrogel dissolves at elevated tempearatures (>40˚C)	Physical cross-linking (Valentin et al., 2017; Ling et al., 2007; Huang et al., 2013; Jang and Nam, 2015) Carbodiimide cross-linking (Jocic et al., 2017)
Alginate Alginate-methacrylate	• Inexpensive • Mild crosslinking conditions • Rapid gelation • Can be used as sacrificial layer	• Hydrogels can dissolve within days due to Ca^{2+} loss • Diffusion dependent gelation that might result in heterogeneous cross-linking • Gelation leads to increase of viscosity and might clog chip	Physical cross-linking (Shin et al., 2007; Valentin et al., 2017; Agarwal et al., 2013; Cagol et al., 2018; Chen et al., 2016; Read et al., 1999; Yajima et al., 2018; Zhao et al., 2009; Bae et al., 2014; Lee et al., 2012; Karamikamkar et al., 2018; Chan et al., 2013) Photo-initiated radical cross-linking (Zorlutuna et al., 2011)
Proteins			
Collagen	• Biocompatible • Provides ligands for cell adhesion • Can be gelled without chemical modification • Enzymatically degradable	• Highly viscous in solution which makes handling difficult	Physical cross-linking (Hsiao et al., 2015; Morimoto et al., 2018; Wang et al., 2013b; Zheng et al., 2012a; Herland et al., 2016; Ma et al., 2016a) Photo-initiated radical cross-linking (Bell et al., 2015)
Gelatin Gelatin methacrylate Gelatin-thiol Gelatin norbornene	• Inexpensive • Low viscosity, easy handling • Enzymatically degradable • Provides ligands for cell adhesion	• Rapid degradation • Weak mechanical properties	Photo-initiated radical cross-linking (Miri et al., 2018; Bertassoni et al., 2014; Aung et al., 2016; Ovsianikov et al., 2011; Zuo et al., 2016; Zhang et al., 2016b; Zhao et al., 2016a) Thiol-ene click cross-linking (Skardal et al., 2015; Li et al., 2017, 2018) Enzyme-initiated cross-linking (He et al., 2016)
Chitosan Chitosan methacrylate	• Anti-microbial activity	• Poor stability that restricts applications	Photo-initiated radical cross-linking (Kufelt et al., 2015)
Dextran Dextran-tyramine	• Biocompatible • Anti-fouling material	• Insoluble in water	Enzyme-initiated cross-linking (Henke et al., 2016)
Silk	• Biocompatible • High mechanical properties		Enzyme-initiated cross-linking (Zhao et al., 2016b; Appelgate et al., 2015)
Fibrin	• Form covalent bond without radicals • Enzymatically degradable • Provides ligands for cell adhesion • Biocompatible	• Enzymes can react with functional groups on cell surface, too	Enzyme-initiated cross-linking (Miller et al., 2012; Kolesky et al., 2016; Nagamine et al., 2011; Bang et al., 2017)
Matrigel®	• Mixture of different natural ECM components • Enzymatically degradable	• Expensive • Unknown composition	Physical cross-linking (Wong et al., 2008; Miller et al., 2012)

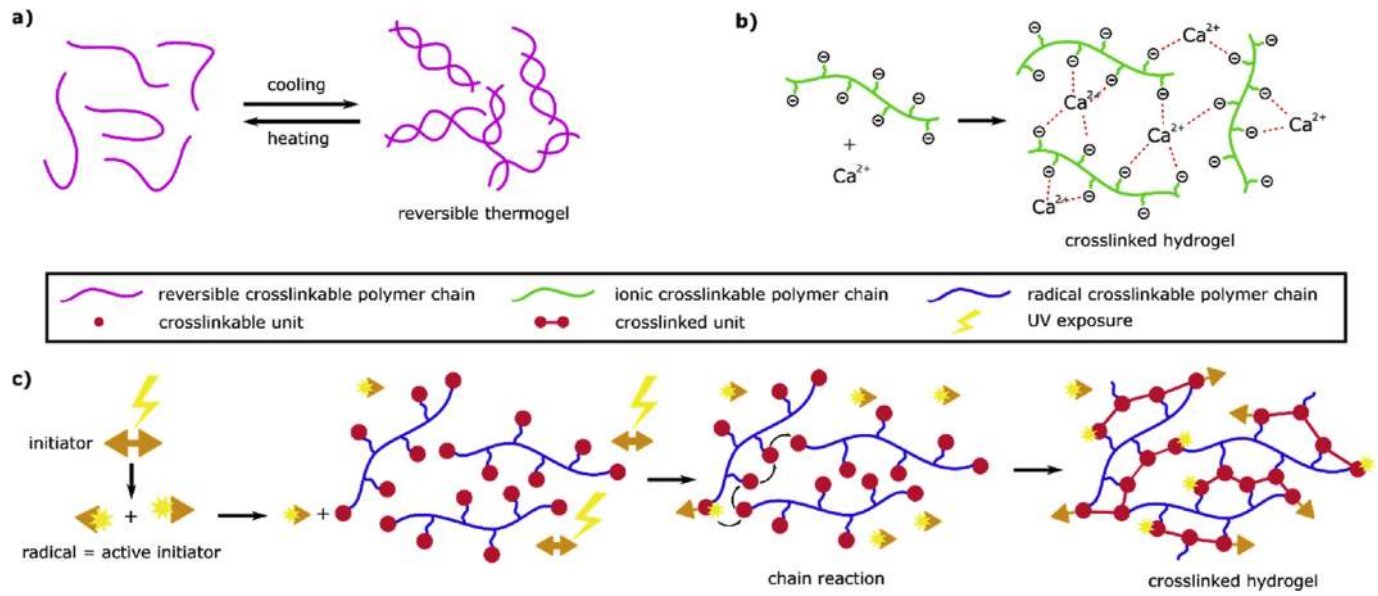

Fig. 3. a) Schematic representation of reversible thermogel formation. b) Schematic representation of ionic cross-linking mechanism with bivalent cations. c) Schematic representation of the radical cross-linking mechanism. UV light activates the initiator generating two radicals, which can react with a functional group of the polymer chain and causing a chain reaction that cross-links many functional groups leading to the formation of the hydrogel.

2009). It is important to control the concentration of the initiator because a too low concentration will lead to a slow polymerisation and in worst case no rigid gel will be obtained at all. Counterintuitively, a higher concentration of initiator does not result in a stronger gel but it will lead to a faster gelation time and thus a final gel with a *lower* mechanical stiffness. An excess of initiator may also react with proteins, change the pH of the gel or damage any encapsulated cells (Mironi-Harpaz et al., 2012) so the concentration of the initiator should always be kept as low as possible. The ideal concentration of initiator will vary for each material combination and must often be determined experimentally.

Covalent radical-free cross-linking

To avoid the risk to damage normal cell functions associated with radical cross-linking, radical-free mechanisms have also been developed. One example is the strain-promoted click reaction which gently encapsulates cells and does not need UV irradiation (DeForest et al., 2009; DeForest and Anseth, 2011). The challenges with this technique is the rather elaborate chemical functionalisation involved. An alternative approach without required functionalisation is the cross-linking of carboxylic acid groups with amine groups (Goubko et al., 2010). This approach lends itself particularly well for naturally derived hydrogels as these often include both these chemical groups. The only requirement for this approach is that the reaction must be activated and this can be achieved by the addition of an activating agent (usually a carbodiimide such as 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC).

Another used approach includes cross-linking of non-functionalised proteins with the enzyme transglutaminase (He et al., 2016) and fibrinogenase (Miller et al., 2012; Nagamine et al., 2011). These reactions have the advantage of forming covalent bonds without the use of radicals or extensive chemical modification reactions. On the other hand, this chemistry is not bioorthogonal (i.e. specific for the cross-linking reaction) which means that the polymer chains may also react with any encapsulated cells. Further, the flexibility of this cross-linking method is reduced as the reaction occurs immediately as the enzyme is introduced into the hydrogel precursor.

Microfabrication techniques

This section will describe different microfabrication techniques that can be used to chemically pattern and spatially define hydrogels with micrometre precision. First, lithographic techniques using either light or an elastomer stamp are described, followed by an introduction to microfluidic techniques to generate hydrogel droplets and fibres. The advantages and disadvantages both related to the technical methods as well as their biocompatibility are covered in the respective subsections below.

UV lithography

UV lithography is the workhorse of the microfabrication techniques developed for the electronics industry (Romankiw, 1997). The set-up comprises a UV light source (fluorescence microscope, UV lamp or light emitting diode (LED) array) that is mounted on top of a plate where the hydrogel precursor solution is placed, with a mask in-between. The hydrogel structures are then defined by exposing the precursor solution to UV light through the mask, Fig. 4. Photolithography can be used for both selective cross-linking (i.e. polymerisation initiated in the exposed areas) and for hydrogel degradation mediated by UV light bond cleavage.

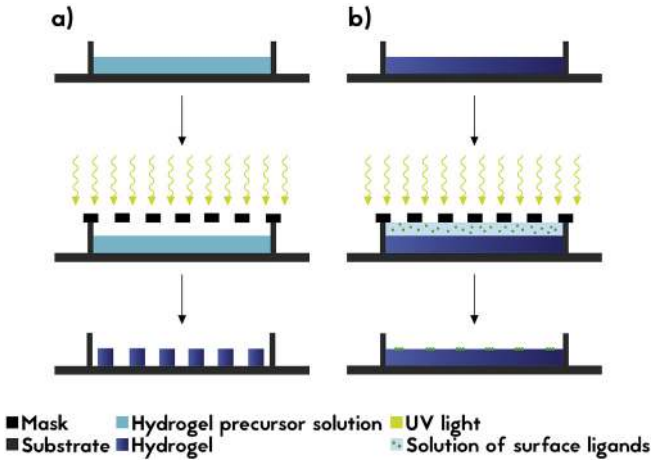

Fig. 4. Schematic image on the use of UV lithography. a) The UV light can be used to define mechanical structures by cross-linking the hydrogel precursor solution. Cells can then be seeded onto these structures. b) The technique can also be used to selectively functionalise the surface to control cell adhesion.

UV lithography may also be used to selectively micropattern hydrogel surfaces by initiating cross-linking reactions between the hydrogel and different ligands placed in suspension on the hydrogel, Fig. 4.

By sequentially exposing the hydrogel through different masks, complex patterns or multilayer structures can be generated just as in the multi-stack processing of the semiconductor industry. The advantages of UV lithography is its simple set-up and high accuracy of the patterns. On the other hand, the cytotoxicity of the photo-initiator radicals is a disadvantage and it is therefore necessary to tune the photo-initiator concentration and UV irradiation dose to avoid cytotoxic effects on the cells (Mironi-Harpaz et al., 2012) or by using long-wave UV (315–400 nm) (Khalil and Shebaby, 2017). Moreover, for thicker samples with limited transparency one can risk to generate a gradient in cross-linking due to an uneven exposure dosage in the vertical plane. Sometimes gradients are wanted and they can be generated horizontally by using a grey-scale mask (Wong et al., 2003) or by sliding the mask across the sample during the exposure (Marklein and Burdick, 2010). When using UV light to both fabricate and chemically pattern a hydrogel, one must be aware that the double exposure to UV light will affect the mechanical properties of the gel until the hydrogel precursor solution is fully cross-linked.

Stereolithography

Stereolithography uses a UV laser to cross-link a photosensitive material (Ikuta and Hirowatari, 1993), Fig. 5. The technology has been developed in recent years to produce objects on the centimetre scale in only a few hours (Seo et al., 2017) and achieve a resolution of 2 μm for polymer-based solutions (Choi et al., 2009). The device consists of a UV laser source, a high-resolution automated *x-y* stage to precisely move the laser beam, and a vertical platform to set the thickness of each layer. A UV sensitive hydrogel pre-cursor solution is placed in a reservoir where the final object is generated layer-by-layer by exposure to UV light according to the shape of the design file (Seck et al., 2010). The x-y scanning of the UV laser is the time-consuming step of the process, thus great benefits have been achieved by implementing digital micro-mirror devices (DMD) to the stereolithography set-up as an alternative to physically moving the light source itself (Sun et al., 2005).

In contrast to the mask-based techniques that are poorly automated and limited in 3D fabrication capabilities, stereolithography offers a versatile strategy to produce 3D scaffolds in a fully automated manner with no need for skilled operators. However, the technique is still not able to fabricate branched 3D structures and microfluidics chips without additional material supports and the use of photoabsorbers to limit the light penetration depth to around 100 μm (Zhang and Larsen, 2017).

Two-photon polymerisation

The two-photon polymerisation technique (2PP) is a method that exploits femtosecond laser pulses, typically in the near infrared (IR) spectrum, for cross-linking photosensitive materials with extremely high resolution (Maruo et al., 1997). With 2PP, it is possible to achieve free-hanging structures without the need of material supports or photo-absorbers. The 2PP device set-up consists of a femto-laser and a high-resolution *x-y-z* stage, Fig. 5. The recent technology development allows for complex hydrogel structures with a feature size down to around 5 μm (Brigo et al., 2017), total construct size of a few mm's (Ovsianikov et al., 2011) and a writing speed up to 50 mm/s (Qin et al., 2018). The 2PP technique offers a more cell-friendly process as it utilises the near IR spectrum for the photo-initiator activation instead of the cytotoxic UV-range. Further, the technique can be tuned for both cross-linking (Ovsianikov et al., 2011) and polymer chain photo-cleavage (DeForest and Anseth, 2011; Tibbitt et al., 2010) even in cell-laden hydrogels (Appelgate et al., 2015).

The ablation impact on cell viability was assessed by S.R. Burtch et al. (Burtch et al., 2018) and they demonstrated that 75% of the dead cells were within 32 μm from the centre of the 28 μm wide ablated channel. This shows that the cytotoxicity of the technique is confined to the processed area. Despite the high resolution and possibility to freely polymerize a hydrogel in 3D, the high price of the equipment, need of specialized technicians and the long processing time are hampering the wide use of this technique. Constructs smaller than one centimetre can require hours or even days to fabricate making it very challenging to ensure maintained cell viability. In order to address these issues, the development of photo-initiators with faster and more efficient activation as well as the integration of technological advancements, are the current focus of research (Kato et al., 2005; Li et al., 2009).

Soft lithography and moulding

The term soft lithography comprises a group of fabrication techniques that uses a stamp, sometimes referred to as a master, to define chemical or structural patterns in another material (here the hydrogel). While the fabrication of the stamp requires UV lithography and is thus costly, it can be used several times in standard chemical laboratories, making soft lithography overall a low-cost fabrication method.

In the first approach, μCP, the stamp is used to create chemical patterns as small as 2 μm (Hynd et al., 2007b) up to 200 μm (You et al., 2014) on the surface of the cross-linked hydrogel. First, the stamp is inked with the desired solution, dried and then placed into contact with the hydrogel surface, Fig. 6. Adjacent and complementary patterns can be formed by repeating the printing steps (Burnham et al., 2006).

Alternatively, the stamp can be used to replicate its inverse into a hydrogel by a process called moulding, Fig. 6. Moulding can, in fact, be performed using a range of alternative stamps where for example, Jocic et al. (2017) created a circular central hollow lumen within a thermostable hydrogel, by using a wire inside a capillary as the stamp. Huang et al. (2013) used the same technique but instead of forming a straight channel, they moulded a spiral channel in an agarose hydrogel using a metal spring. The limiting aspect on what structures that can be defined using moulding is the removal of the solid master after the hydrogel cross-linking, and here the use of a simple stamp as shown in Fig. 6 is beneficial as it can be lifted straight up.

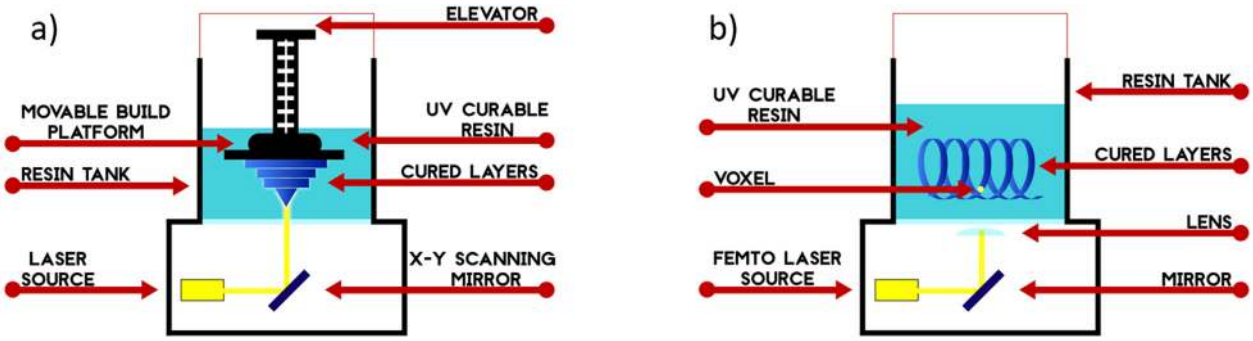

Fig. 5. Schematic image of two other lithographic techniques. a) Stereolithography and b) Two-photon polymerisation (2PP).

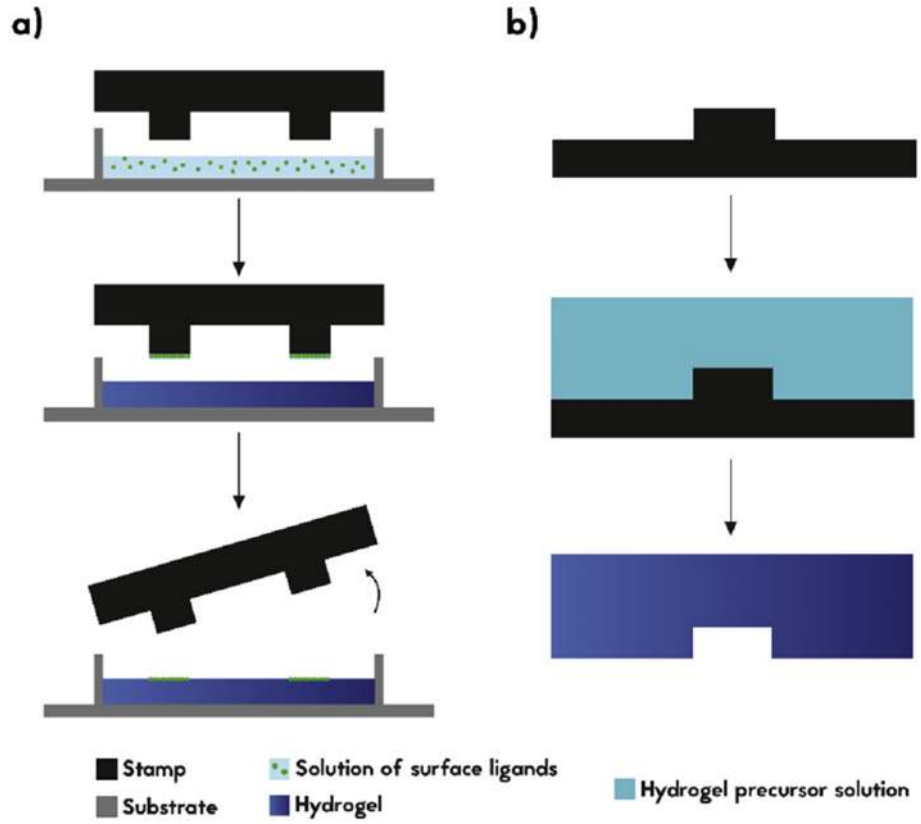

Fig. 6. Schematic images of two methods within the soft lithography fabrication group. a) Here the stamp is used to transfer a chemical pattern onto a hydrogel surface (μCP) and in b) the micropatterned structures are used for moulding of the hydrogel.

To allow for more complex structures, the final system can either be moulded in two independent halves which are sealed together or a sacrificial mould that can be dissolved can be used (Bertassoni et al., 2014). The challenge with using a sacrificial mould is to tune the dissolution of the sacrificial material to the cross-linking time of the hydrogel to prevent premature dissolution of the mould, which would leave incomplete structures. Despite the advantages of moulding, crucial limitations affect the quality of the resulting scaffolds such as low feature resolution (≥100 μm) and the difficulty to handle the final hydrogel constructs.

Microfluidic techniques

By introducing the hydrogel precursor solution into a microfluidic chip, one immediately gains more control over the final dimensions and resulting stability. For example, Wang et al. created a channel in a hydrogel and Ma et al. demonstrated control of different hydrogel structure thicknesses by using a microfluidic chip with a pneumatic membrane serving as the top of the microfluidic channel (Wang et al., 2013b; Ma et al., 2016a). In both examples, the hydrogel precursor solution was introduced into the channel and then the pneumatic lid was either lowered or lifted before the polymer cross-linking occurred. A. Miri et al. demonstrated a similar approach where a flexible lid was lowered into the channel mechanically instead of pneumatically. After cross-linking the hydrogel to the defined thickness and removing the non-cross-linked hydrogel precursor solution, additional layers could be formed by adjusting the membrane height and introducing another hydrogel precursor solution (Miri et al., 2018). Here, stereolithography was used as the cross-linking source, which added an extra level of complexity in what shapes that could be defined.

An interesting approach to fabricated microfluidic channels with circular cross-section in a hydrogel is the viscous fingering method, Fig. 7b. In this method, a microfluidic channel is first filled completely with the hydrogel precursor solution. Parts of this solution is then removed by perfusing cell media centrally in the microchannel, resulting in a hydrogel layer on the channel walls with a circular opening in the centre (Bischel et al., 2012). The technique offers good reproducibility with a hydrogel layer thickness of around 700 μm. The technique does however require substantial optimisation and expertise to generate a uniform structure and to avoid the complete removal of the hydrogel precursor solution during perfusion (Herland et al., 2016).

Microfluidic systems can also be used to spatially confine hydrogel precursor solutions using surface tension, Fig. 7c. The confinement is achieved by properly designing microstructures in the microfluidic system such as micropillars that restrict the liquid spread in the channel (Bang et al., 2017). Typically, the hydrogel pre-cursor solution is introduced and cross-linked in a centre channel with adjacent microchannels used for perfusion of cell-specific media for the culture. A more flexible approach, although slightly more complicated in its operation, is to use laminar flow patterning to define the widths of the hydrogel section (Kim et al., 2007).

Microfluidics has also become established as reliable alternatives to conventional bulk emulsification (Read et al., 1999; Levee et al., 1994; Lim and Sun, 1980) and extrusion (Chang et al., 2008; Sugiura et al., 2008) for the generation of round and linear cell-laden hydrogel constructs (here termed microgels and microfibers). In two-phase microfluidics, the structures are commonly formed by the laminar flow of two immiscible liquids; a disperse phase (forming the final constructs) and a continuous phase, as they meet at a junction, Fig. 8. Forces seeking to minimize the interfacial area create instabilities between the meeting immiscible fluids, which will either lead to a co-flow or a drop-making regime depending on the properties of the fluids and the applied flow rates (Utada et al., 2007; Guillot et al., 2008). The size of the generated constructs is determined by the dimensions of the microfluidic system in

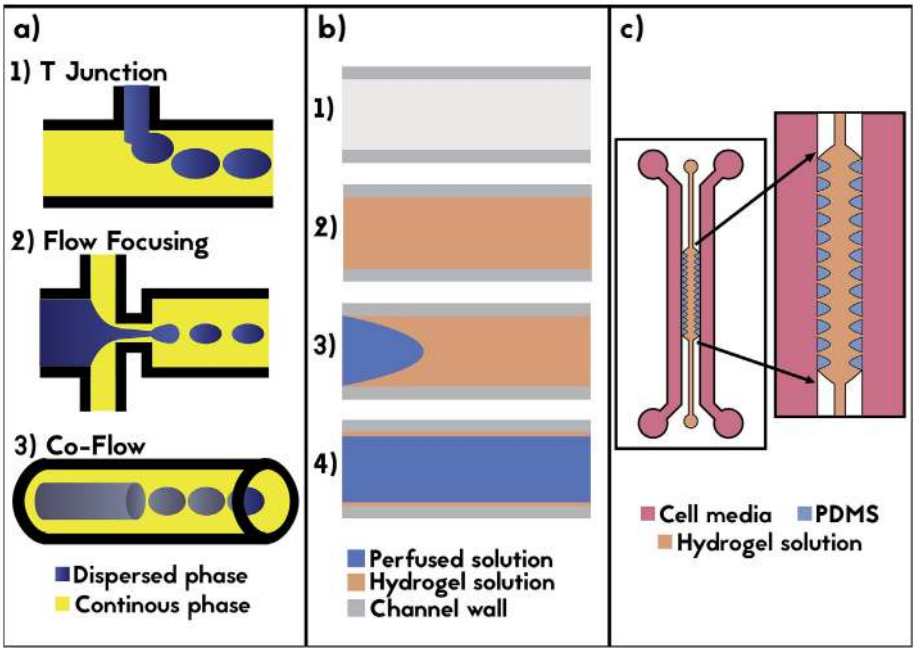

Fig. 7. Schematic drawings showing some of the microfluidic methods that can be used to define hydrogel cell culture scaffolds. a) Three different methods to generate hydrogel droplets. b) A PDMS channel was filled with collagen and a microfluidic channel was formed inside with the method of viscous fingering (Herland et al., 2016). c) The hydrogel is confined in the centre area of the microfluidic chip via microposts (Bang et al., 2017).

combination with the fluid properties and the applied flow rates (Takei et al., 2010). Two-phase microfluidic devices can produce well-controlled spherical microgels (Carreras and Wang, 2017; Hong et al., 2012; Kumachev et al., 2011; Rossow et al., 2012; Shintaku et al., 2006) in the range of 20–100's of μms in diameter, as well as cylindrical and hollow fibres from aqueous jets (Onoe and Takeuchi, 2015) of similar diameter but up to a few metres in length, without the need for masks and masters. Early set-ups for microgel production used needles (Sakai et al., 2008), or micro-nozzles extrusion where a cell-laden alginate solution was dripped into a calcium chloride solution to produce either microcapsules (Sugiura et al., 2005) or microfibers (Sugiura et al., 2008). The challenge when producing these types of structures is to ensure homogeneous cross-linking and one interesting approach was demonstrated by Shintaku et al. where they produced differently shaped beads that were uniform in size by fusing alginate droplets with $CaCl_2$ droplets downstream in the microchannel (Shintaku et al., 2006) to control the timing of the reaction more carefully.

To generate droplets on the sub-μm scale, one needs to turn to step emulsification, where droplets are generated from an abrupt change in the microfluidic channel's height (Li et al., 2015). Step emulsification has also been used to increase the throughput of the droplet production compared to a flow focusing devices. Such small droplets are however not common to use for cell-laden constructs but used as 3D culture scaffolds for cell seeding (de Rutte et al., 2019). During fibre production, the outer phase, known as the continuous phase, is generally a lower-viscosity aqueous solution whereas in microgel production it is typically a non-toxic oil such as fluorinated oil, corn oil, mineral oil or silicone oil with added surfactants to provide droplet stability by reducing the surface tension between the phases (Shin et al., 2007; Guillot et al., 2008; Anna et al., 2003; Umbanhowar et al., 2000). Microgels may also be formed in water-water emulsions by taking advantage of viscosity differences between the two aqueous phases (Moon et al., 2016) although this application is not that common.

The three most common microfluidic device geometries for the production of droplets and jets are flow-focusing, co-axial and T-junction, where the T-junction geometry is the most commonly used design, Fig. 7a. The devices can be fabricated by embedded capillaries (Utada et al., 2007), PDMS structures fabricated via soft lithography (Kang et al., 2010), low-cost alternatives as pipette tip-based microfluidic devices (Li et al., 2017), and recently through 3D printed modules (Morimoto et al., 2018). All of these methods have enormous capabilities regarding throughput but the challenge lies in handling and monitoring the constructs onwards in the culture. For this, microfluidic devices (Carreras and Wang, 2017; Kim et al., 2012) or selectively patterned surfaces (Li et al., 2011) have been developed as possible approaches.

Advantages of microfluidics for the production of hydrogel cell culture scaffolds include high monodispersity in the constructs, automation to a continuous process and great reagent reduction as only the material to be used for the final structures are used during fabrication. Other benefits of microfluidic formation of fibers include the ability to produce hollow and multilayered microfibers (Lee et al., 2009b). The microfluidic approach in its simple design and operation as described here, is however limited to the production of exclusively spheres, capsules, fibres or tubules. This means that although the fabrication of the final microgel constructs are simple, the fabrication of the necessary microfluidic system can be both advanced and time-consuming. Moreover, fluid

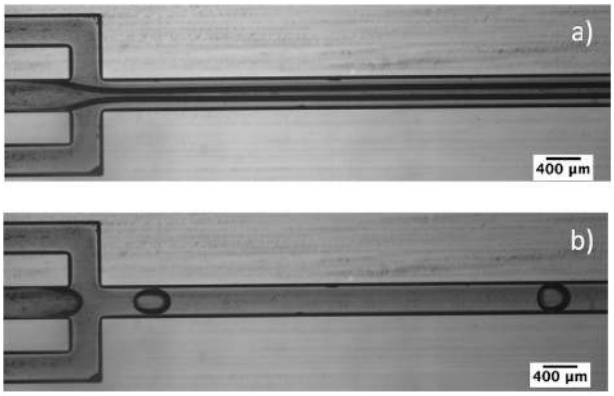

Fig. 8. Optical images of a co-flow microfluidic system operated in two different modes; a) fibre production and b) droplet generation. By varying the capillary number of the system (e.g. by varying the flow rate), the same microfluidic system can be used for both production methods.

dynamics is heavily affected by the flow rate, viscosity, density of the different phases, and interfacial tension, surface chemistry and device geometry (Nie et al., 2008; Nunes et al., 2013). This means that typically customized microfluidic systems need to be designed, fabricated and optimised for each desired application. In summary, this means that this fabrication method is very efficient for operation and microgel production, but it may require a rather extended optimisation process until being fully operational.

Applications of microfabricated hydrogels

In this section, we will discuss some examples from the literature where microfabrication techniques have been applied to develop hydrogel based cell culture scaffolds for organ and disease models. This is not an exhaustive list but it is intended to show the possibilities with combining microfabrication techniques and biomaterial research and to serve as inspiration for development of other *in vitro* models with high *in vivo* resemblance.

Hydrogels as device material

When using a hydrogel for forming a device geometry rather than a cell scaffold that the cells should be able to penetrate, PEG based hydrogels is a specifically good choice as it prevents protein adsorption and does not support cell adherence (Koh et al., 2003; Zhu, 2010). Koh *et al.* demonstrated proof-of-concept of using PEG-DA to form microwells (20 μm × 20 μm × 10 μm well dimensions) on glass slides, which captured fibroblasts or hepatocytes, by defining and controlling regions that resist cell adhesion (PEG-DA walls) and promote cell adhesion (the glass slide). Lee et al. fabricated a PEG-DA microwell arrays (200 μm × 200 μm × 140 μm well dimensions) on double-layered polystyrene (PS) nanofiber scaffolds to culture spheroids. The top layer consisted of bare hydrophobic PS and served as cell adhesion layer, whereas the bottom layer PS nanofibers were coated with anti-albumin for the *in situ* detection of albumin. The hydrogel array, together with the bare PS, promoted the formation of uniform human liver derived cells (HepG2) spheroids to a desired size of 200 μm to prevent hypoxia and malnutrition. Moreover, this system permitted simultaneous detection of albumin secretion by the spheroids, a marker of liver cells function (Lee et al., 2011). The role of the hydrogel material was to hold together the two PS nanofiber layers. Moreover, by controlling the size and material of the microwells, and hence their physical and chemical composition, the authors were able to prevent cell adhesion to the PEG hydrogel microwell walls and control the spheroid size.

Although it may seem attractive to fabricate the cell culture platform in the hydrogel itself it must be remembered that utilising hydrogels as device material is extremely challenging due to swelling, degradation and lack of structural support of these materials. Already in 2007 when Y. Ling et al. (2007) demonstrated proof-of-concept of an all-hydrogel cell-laden microfluidic chip, they discussed the challenges such as nutrient depletion, waste removal from the culture matrix and cell viability. Other practical aspects such as rapid dehydration and low mechanical properties of hydrogel-based structures were highlighted by Cuchiara et al. (2010). Cuchiara et al. solved it in their system by embedding the PEG hydrogel within a stiffer material (PDMS) as casing, serving both as the mould during fabrication and subsequently structural support to facilitate tubing connections and device manipulation. The approach to use a solid casing material was employed by Fu et al. as well when they prepared PEG microstructures, using UV lithography in a pre-assembled chip, with a U-shape geometry for cell entrapment and spheroid formation. The authors achieved heterotypic spheroids by loading two cell types (HepG2 and 3T3) sequentially, for a more *in vivo* like model. Moreover, long-term culture and assessment of cell metabolic activity when exposed to doxorubicin, a chemotherapeutic agent, was achieved on the same chip. The cells were more resistant to doxorubicin if they were allowed to grow as spheroids compared to monolayer cultures, addressing the benefits gained when using microfabricated cell culture structures (Fu et al., 2014).

On-chip spheroid formation or cell encapsulation, culture and analysis on a single device reduces steps and easies the handling as well as it reduces the risk of losing samples or making mistakes.

The examples listed above describe whole systems fabricated in hydrogel but the material can also be used as a structural component of a 3D cell culture scaffolds as demonstrated by Griffin et al. that produced mm-scaled scaffolds from μm-sized hydrogel droplets (Griffin et al., 2015). The constructs were prepared by annealing the droplets into large-scale scaffolds via enzymatic reactions leaving a cell-friendly material with high porosity.

Chemical patterning of hydrogel scaffolds

Chemical patterning of hydrogels to control surface chemistry at the micrometre level permits mimicking native tissue architecture. By patterning biochemical cues on the surface one can spatially control cell adhesion, Fig. 9. UV lithography is specifically well suited for chemical 2D modifications as it can be applied to scaffolds with a complex topography. First report in literature using UV lithography for this purpose was published in 2006 by Hahn et al. where they showed the formation of 40 μm wide lines of cell adhesive RGD peptide (ACRL-PEG-RGDS) patterned on a PEG hydrogel (Hahn et al., 2006). Just like in the work by several other research groups (Wade et al., 2015; Moon et al., 2009; Gu and Tang, 2010) the result was that the seeded cells selectively adhered to the areas patterned with the RGD peptide. The minimum

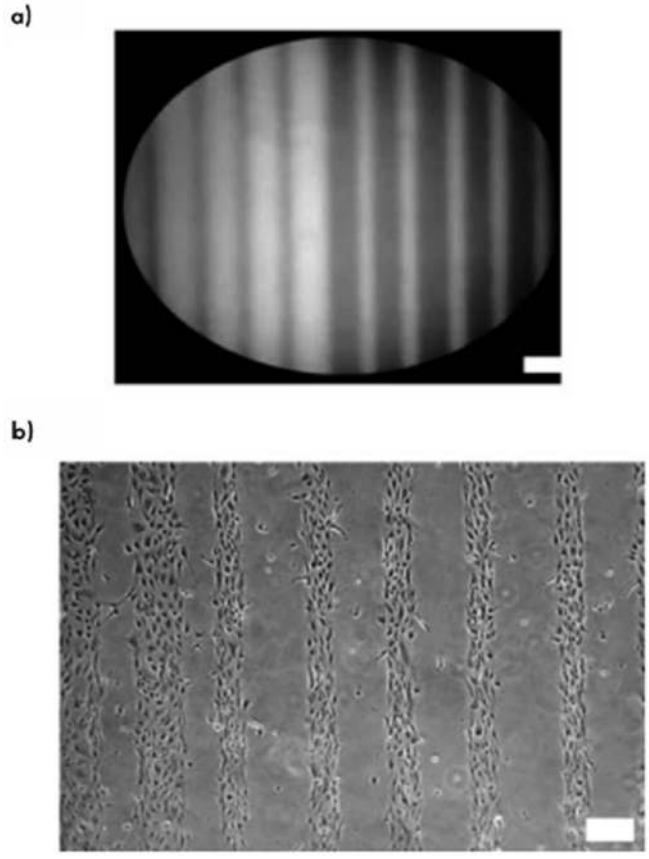

Fig. 9. Chemical pattern on a hydrogel surface controls the cell adhesion.
a) Fluorescent image of PEG-RGDS lines patterned with UV lithography on a PEG-DA hydrogel.
b) Optical image showing selective adhesion of HUVEC cells to the RGD lines. Scale bars = 200 μm.
Reprinted from (Moon et al., 2009) with permission.

resolution that can be obtained is determined by the technique used and which molecule that is patterned. To date, Larsen et al. holds the record using projection lithography to pattern 1 μm narrow fibronectin stripes onto PEG-DA (Larsen et al., 2014).

In vivo, both physical and chemical cues control different cell processes. To study the interplay between these, Wade et al. linked RGD peptides to electrospun hyaluronic acid nanofibrous hydrogels (Wade et al., 2015) where the peptides stimulated the cells via chemical cues and the nanofiber structure and its alignment communicated via physical cues. The authors noted that the adhesion and morphology of the 3T3 fibroblasts seeded on the scaffold followed the RGD patterns although their orientation followed the nanofibers direction, independent of the RGD pattern. They concluded that the mechanical cues had a stronger effect than the chemical cues in this specific cell/matrix combination on these cells (Wade et al., 2015). In the examples presented above, the peptides are selectively linked to specific areas on the hydrogels during the fabrication, resulting in a static pattern during the cultures. Goubko et al. have demonstrated a "dynamic pattern" by linking RGD peptides with a photo-labile caging group onto the hydrogel surface. The RGDs can then be selectively activated by removing the caging groups by UV exposure (Goubko et al., 2010), allowing for a flexible surface patterning technique.

3D chemical modifications can also be achieved by soaking the bulk hydrogel in a peptide solution and consecutively exposing selected areas to UV light through a mask (DeForest et al., 2009; DeForest and Anseth, 2011). Using this approach, DeForest et al. used patterning of collagenase-sensitive peptide sequence (DiFAM), for *in situ*, read-out of cellular activity, rather than controlling cell adhesion as the example described above.

They micropatterned the DiFAM peptide to a four-arm PEG tetra-azide hydrogel throughout its bulk. The feature of the peptide is that it fluoresces upon enzymatical cleavage by collagenase excreted by the cells. The fluorescent signal was then used as a sophisticated method to monitor the cellular protease activity within the hydrogel in real time (DeForest et al., 2009). However, the challenge in this approach is to remove all non-bound peptides before seeding the cells to obtain clearly defined functionalised areas.

UV lithography has several advantages such as high resolution, high throughput, possibility to work on surfaces with uneven topography and deep penetration depth allowing for bulk modifications. The disadvantage related to bulk modifications is that all the exposed material will be modified so for selective modifications in the bulk, 2PP is a better option as this technique only initiates reactions at the focal voxel (Culver et al., 2012). Demonstrating this, J. Culver et al. accurately replicated a blood vessel network tissue by guiding the growth of human umbilical vein endothelial cells (HUVEC) with patterned RGD peptides on an otherwise inert PEG-based hydrogel. The patterning can also be combined with microfabrication to simultaneously provide chemical and mechanical cues to the cells. This was demonstrated by M. Skyler-Scott et al. when they patterned the cell adhesion molecule P-selectin on a previously fabricated channel network to guide and trap HL-60 cells (Skylar-Scott et al., 2016). These two methods clearly show the potential of obtaining more tissue like scaffolds by tailoring their properties on the micrometre scale. In the second example, both the chemical and the mechanical properties were modified as the same chemical pathway was used for hydrogel cross-linking and functionalisation. If this is not desirable for the application, one can utilise the method of μCP instead. Most commonly, this technique is used to control cell attachment through the patterning of peptides and proteins directly onto the hydrogel surface, either via adsorption (You et al., 2014; Castaño et al., 2014; Beckwith and Sikorski, 2013), non-covalent streptavidin-biotin interaction (Zhang et al., 2010; Hynd et al., 2007a, 2007b; Agarwal et al., 2013) or via covalent cross-linking (Sarker et al., 2018; Hsiao et al., 2015; Greiner et al., 2014). Even though the biotin-streptavidin linkage relies on non-covalent interactions, it is one of the strongest non-covalent bonds ensuring that the surface modification remains stable over the culture time (Hynd et al., 2007b). Moreover, many polymers and proteins labelled with streptavidin and biotin are commercially available whereas the other techniques typically require complex or low biocompatible chemistries for their functionalisation.

The μCP technique has one major advantage over UV lithography and that is that the stamp simultaneously can be used to chemically pattern the hydrogel as well as to mould structures into it. You et al. formed heparin hydrogel microwells in this method by inking their PDMS stamp, containing arrays of 200 μm diameter posts, with collagen I before printing into a PEG-DA-heparin hydrogel precursor solution and cross-linking via UV exposure (You et al., 2014). This resulted in 200 μm microwells coated with collagen I at the bottom that the hepatocytes adhered to. Once the hepatocytes had adhered, fibroblasts were seeded and adhered to the non-modified heparin walls. The resulted co-cultures where highly functional, producing higher levels of albumin, a liver function marker, than hepatocytes in monoculture. Gobaa et al. used a similar approach but instead of just inking their stamp with the same molecules throughout, they used a DNA spotter to prepare the silicon stamp with different protein concentration and combinations. In this manner, they showed the production of 2016 PEG hydrogel microwells on a standard glass slide (18 cm^2) and 288 microwells in each 12-well--plate well with individual biomolecules added at the bottom. Subsequently, cells where seeded and captured in the microwells. This approach permitted the high-throughput analysis of cell fate and differentiation of primary human mesenchymal stromal cells (hMSCs) in relation to different protein concentrations and hydrogel stiffnesses (Gobaa et al., 2011).

Microfabricated cell-laden hydrogel constructs

One of the first examples of cell-laden scaffolds was demonstrated by Liu & Bhatia in 2002 (Liu and Bhatia, 2002) as a combination of moulding and UV lithography. In their work, they encapsulated HepG2 in a PEG-DA hydrogel as proof-of-concept to form hepatic tissue constructs by crosslinking cell-laden layers, one after the other, resulting in three layers of PEG-DA containing cells. The authors carefully investigated the effect of UV exposure and photo-initiator concentration on the final pattern resolution and cell viability. They could demonstrate that UV exposure itself did not reduce cell viability whereas the photo-initiator did. In their work they even showed that the photo-initiator resulted in cytotoxicity in a dose-dependent manner in the absence of UV light. As mentioned above, it is the radicals formed by the activation of the photo-initiator that is toxic and it clearly shows that the choice of photo-initiator is one of the most important aspects to consider when preparing cell-laden constructs using UV lithography. The benefits with encapsulating the cells in the hydrogel sheets were that the authors could form more complex structures of the cells and were no longer limited to 2D cultures.

One use of cell-laden hydrogels is to study cell migration related to matrix properties. Kloxin et al. prepared a PEG hydrogel slab which could be selectively photodegraded upon UV light exposure (Kloxin et al., 2009). hMSCs were encapsulated within the hydrogel and initially exhibited a rounded morphology due to the dense hydrogel network. The hydrogel was then degraded in selected areas using UV lithography or 2PP and the cells were observed to spread because of the decreased network density. Similarly, Khetan & Burdick studied the proliferation of hMSCs in a HA hydrogel (Khetan and Burdick, 2010). First, the HA hydrogel containing cell adhesive peptides and enzymatically degradable oligopeptides was formed by addition reactions. The hydrogel was then exposed to UV light through a photomask to form non-degradable kinetic chains in the exposed areas, increasing its mechanical properties in these areas (~15–18 kPa). This prevented the encapsulated hMSC's in the stiffer areas to spread and made then undergo adipogenesis, while the cells encapsulated in the non-exposed areas (with Young's modulus ~6 kPa) underwent osteogenesis. It was surprising to see that the bone forming cells preferred the softer matrix areas and the authors

hypothesise that it was dominantly the ability of the cells to spread in the matrix that determined the cell fate. These findings stress that the substrate properties such as elastic modulus, adhesive ligand density, degradability and crosslinking strategy collectively act on the cell fate (Khetan and Burdick, 2010).

A great variety of cell types including fibroblasts (Yamada et al., 2012), endothelial cells (Zuo et al., 2016; Takei et al., 2010; Lee et al., 2009b), fibrosarcoma (Kang et al., 2011) and hepatocytes (Yajima et al., 2018) have been encapsulated within microfluidic-based alginate fibers where the continuous phase initiate gelation of the hydrogel pre-cursors solution through Ca2+ diffusion at the interface. Interesting examples include the encapsulation of pancreatic islets within collagen-alginate composite by Jun et al. for the production of insulin *in vivo* in mice without eliciting an immune response (Jun et al., 2013) as well as similar work by Onoe et al. (2013).

Photo-polymerisation is a popular method for cross-linking microgels as it allows for on-demand gelation of the constructs either on or off-chip. Photo-crosslinked microgels have been predominantly produced from PEG (Hati et al., 2018; Li et al., 2011; Xia et al., 2017) and functionalised gelatin such as gelatin methacryloyl (GelMA) (Li et al., 2017) where Zhao et al. demonstrated the addition of growth factors into the GelMA matrix (Zhao et al., 2016a). This addition resulted in a gel where the encapsulated bone marrow-derived mesenchymal stem cells exhibited significant osteogenesis both *in vitro* and *in vivo*.

Similar to other cell-laden 3D cell culture scaffolds, microgels and hydrogel fibres should possess sufficient rigidity for handling and assembly combined with high biocompatibility for cell functional expression. One shortage of the current cross-linking strategies used is lack of this combination and one recent approach to address this is the novel functionalisation of alginate through covalent addition of functionalised PEG. Introducing PEG would improve the mechanical properties of the scaffolds (Passemard et al., 2017).

Defined co-cultures from hydrogel modifications

An extremely powerful method of microfabricated hydrogel scaffolds is the potential to produce spatially defined co-cultures to study cell-cell interaction and cooperation. This can either be achieved by fabricating microstructures that selectively positions the cells via physical barriers or to prepare chemically modified hydrogels or microfabricated cell-laden hydrogel constructs where the position of the different cells are controlled during its fabrication. An approach using the combination of chemical and physical patterning was discussed above (You et al., 2014).

2D co-cultures have been demonstrated by several groups either using UV lithography (Otsuka et al., 2013) or μCP (Beckwith and Sikorski, 2013) to prepare chemical patterns on a hydrogel scaffold where different cell types can be seeded successively. Goubko et al. used their technique with the photo-caged peptides described above to prepare a fibroblast/endothelial co-culture. First, they allowed fibroblasts to selectively adhere to the RGD patterned areas on a HA gel. Upon a second UV exposure step, the whole HA gel became cell-adhesive and endothelial cells were seeded and adhered onto the whole structure (Goubko et al., 2014). The benefit of this approach is that the first cell type is not constrained in its original area but is able to migrate across the whole hydrogel surface to freely interact with the other cells. Based on their first work in 2011 when Lee et al. showed the suitability of electrospun polystyrene nanofibers combined with PEG-DA as device material for cell culture wells (Lee et al., 2011) the authors demonstrated in 2014 how these scaffolds could be used for co-cultures by simply stacking the porous sheets, containing different cells, on top of each other. By co-culturing fibroblasts growing in the first layer with HepG2 cells in the second layer, it was shown that the secretion of albumin was increased by the presence of fibroblasts compared to a mono-culture of HepG2 (Lee et al., 2014).

Hammoudi et al. presented a co-culture system comprising ligament fibroblasts and marrow stromal cells defined as a cell-laden mixture of poly (ethylene glycol) fumarate and PEG-DA polymer with the aim to model stem cell interactions with injured tendon/ligament tissue (Hammoudi et al., 2010). The hydrogel construct was prepared by UV lithography where the two different cell types were patterned through complementary photo-masks in consecutive steps. The dimensions of the features were in the order of 0.9–3 mm in width and the co-culture constructs were extracted from the microfluidic device after fabrication for culture in traditional cell culture plates. When co-cultures are prepared, there could be advantages of using more than one type of material to provide conditions that meet the needs of each different cell type as different cells often have different preferences on the chemical and mechanical properties of the scaffold. Cell-laden co-cultures including even three cell types were prepared by P. Zorlutana et al. using stereolithography and two different hydrogels (Zorlutuna et al., 2012). In this work, the scaffold consisted of multilayer structures where the layers were made of two different hydrogel/cell composites. The first layer comprised PEG-DA mixed with oxidized alginate functionalised with RGD peptides where skeletal muscle myoblast cells and primary hippocampus neurons were embedded and the second layer comprised adipose-derived stem cells encapsulated in PEG-DA. By controlling the spatial organisation of these two cell types, the system could be used to study the cholinergic functionality of the neurons in the presence of the myoblast cells.

Preparing 3D co-cultures is to take one more step towards *in vivo* like culture conditions. For this, Ma et al. used cell-laden collagen to replicate a liver lobule (Ma et al., 2016b). To fabricate the cell-laden structures on-chip, they used a dynamic fabrication approach that integrated a pneumatic membrane along the channel of the microfluidic platform and by bending and lifting the membrane they are able to control the channel height during the injection and cross-linking of the material, thereby controlling the final hydrogel thickness and obtaining structures with feature sizes holding biological relevance. Using the same method, they also showed the fabrication of multilayer structures with different hydrogels in several different sizes and shapes (Ma et al., 2016a). The micrometre structures were directly fabricated inside the chip, thereby removing any further substrate handling steps and ensuring a well-controlled environment for both the fabrication and the following biological studies. Another approach to use a microfluidic chip to fabricate cell-laden constructs is the stop flow lithography (SFL). In this technique, a precursor solution containing cells is introduced through a microfluidic device while UV light pulses are applied through a mask so that not all of gel solution is cross-linked. The formed constructs can then be recovered at the outlet of the device for further handling and culture (Panda et al., 2008). Co-cultures can also be generated in microgels by using co-flow geometries where different cell suspensions are introduced at the droplet generation site, jointly forming a co-culture droplet. Stem cell multicellular spheroids (Chan et al., 2013) and core-shell “organ in a droplet” hepatic models (Chen et al., 2016) are examples of structures that have been generated with this method. In their work, Chen et al. demonstrated that liver-specific functions such as albumin secretion and urea metabolism, both exhibited higher activity in the microtissues as compared to that in hepatocytes alone, again addressing the importance of adding more than a single cell type in the culture. The benefits with performing the co-cultures in a microgel format is that nutrient supply and waste removal is not limited as the dimensions are typically below the diffusion limit. Further, this format is still compatible with standard cell proliferation assays, which could be beneficial.

Similar to microgel co-cultures, multiple cells can be integrated into fiber constructs, for example by encapsulating one cell type in the middle layer and another cell type in the outer layer. From the cellular aspect, co-cultures set up in a fiber format is the same as when set up in a microgel format, but from the user's perspective it can be beneficial to work with a fiber as it is much easier to manipulate. Zuo et al. demonstrated one such co-culture by encapsulating vascular endothelial cells and osteoblasts to generate a biomimetic osteon-like structure (Zuo et al., 2016). During the incubation period, both cell types exhibited robust growth and tissue-like

up-regulated gene expression when cultured in the GelMA-alginate composite. It is also possible to prepare fiber-based co-cultures by seeding cells on the outside of a cell-laden fiber as demonstrated by Yajima et al. where they assembled alginate-barium fibers with densely packed hepatocytes inside and superficially seeded vascular endothelial cells into a vascular network-like system mimicking the hepatic lobule (Yajima et al., 2018). The bundled and packed fibers were later incorporated into a perfusion chamber to evaluate cell viability and functions, and monitor oxygen consumption. Most commonly, transversal co-cultures are prepared but Kang et al. have demonstrated longitudinal co-cultures in a fiber by modifying the chemical and cellular composition during its production using a set-up comprising a digital, programmable flow controller (Kang et al., 2011). Hepatocytes and fibroblasts were encapsulated either individually or as parallel co-cultures. Similar to previous results, the authors report the fraction of viable cells in hepatocyte-only cultures decreased throughout the 5-day experiment, whereas the cells in the co-culture better maintained their viability. In short, there are a multitude of reports in the literature demonstrating improved cell viability and function in co-cultures compared to single cultures, irrespective of the choice of platform. Deciding of whether to perform the co-culture in a slab format, microgel format or fibre format is more a matter of preference of the users.

Perfused models

Most commonly, microfluidic channels are used to model the vasculature and to study shear stress effects and soft lithography is often the chosen technology to build the structures. Fig. 10 shows three different examples on how the vasculature models can be prepared. In general, there are two approaches when forming microfluidic channels inside hydrogels; either one can take the engineering approach and define where the channels should be formed during the fabrication step or one can seed cells inside a hydrogel, provide the right chemical and physical cues and rely upon spontaneous angiogenesis. In 2012, Zheng et al. casted a pericyte- and smooth muscle cell-laden collagen I solution inside a PDMS casing to obtain a channel network that could be connected to external pumps, taking the engineering approach. Endothelial cells were subsequently seeded on the inside of the channels to provide a model to study angiogenesis and factors involved in thrombosis (Zheng et al., 2012a). The channels prepared in this work had a square cross-section which may generate heterogeneity in cell seeding (Green et al., 2009) and shear stress distribution (Lu et al., 2004) and in turn affect cell behaviour and tissue maturation. This issue was addressed by J. He et al. that prepared circular microchannels by assembling two partially cross-linked hydrogel layers containing semi-circular channels that were moulded from a master fabricated through stereolithography (He et al., 2016). The system was used to demonstrate perfused culture of endothelial cells as a valid strategy to model the vasculature. Perfused systems with circular cross-sections have also been demonstrated by Miller et al. that fabricated a 3D sacrificial template made of carbohydrate glass filaments and used it to study angiogenesis by seeding endothelial cells in the channels obtained after dissolving the filaments in water (Miller et al., 2012). The combination of a sacrificial structure with a hydrogel casting allowed the move from a 2D to a 3D system to investigate blood vessel junction formation even between vessels at different layers. Moreover, it paved the way to study thick and densely populated 3D cell structures where the nutrient supply and waste removal was secured via the perfusable network. A method to generate circular microchannels without using sacrificial moulds was demonstrated by Herland et al. where they realised a co-culture vasculature model prepared in a collagen matrix (Herland et al., 2016). The system was used to identify the contribution of pericyte and astrocyte cells (cultured in the cell-laden matrix) respectively to the neuroinflammatory response in a blood-brain barrier model (mimicked by endothelial cells cultured on the inside of the channel walls).

Complex vasculature networks can also be formed from simple self-organisation processes if cells are seeded into biomimetic scaffolds supplied with the right combination of mechanical and chemical stimuli, such as shear stress and growth factors (Moya et al., 2013; Osaki et al., 2018; Kim et al., 2015). Bang et al. used this approach to generate a vascular network by angiogenesis in a cell-laden fibrinogen/thrombin hydrogel solution confined by microposts in the centre of a multichannel microfluidic system (Bang et al., 2017). Endothelial cells and fibroblasts were introduce on the left side of the physically confined hydrogel and a

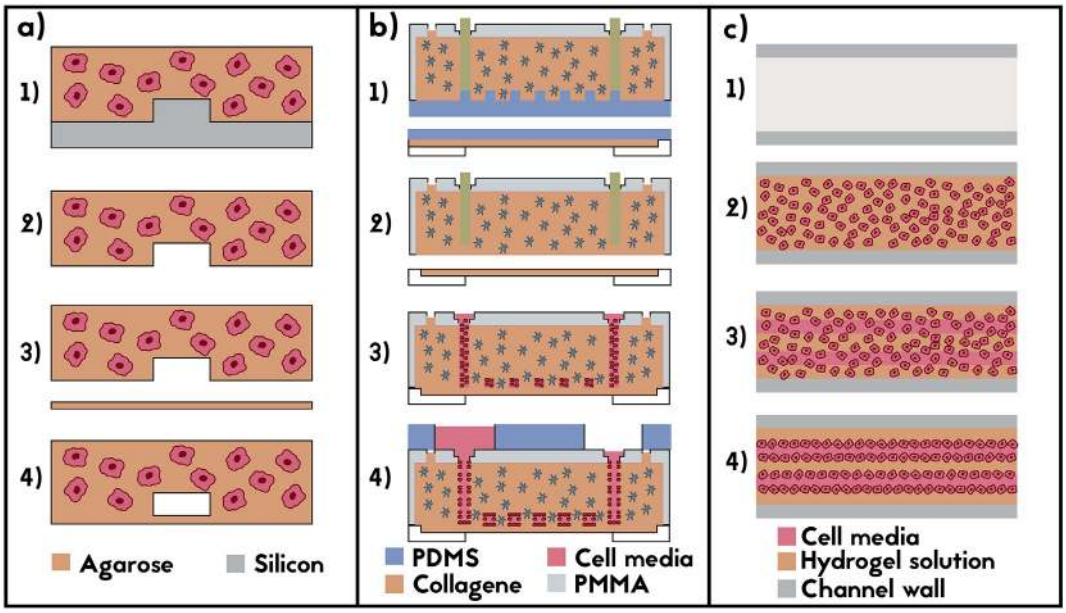

Fig. 10. Some examples from literature on the fabrication of biomimetic perfused systems. a) Cell-laden agarose is moulded on a stamp and later bonded to an agarose plate to form a microfluidic channel (Ling et al., 2007). b) Cell-laden collagen is poured into a mould including defined areas for microchannels to be formed. This is an example of the "engineering approach" to obtain a vasculature model. Endothelial cells are seeded into the collagen channel and the system was used to study angiogenesis (Zheng et al., 2012b). c) A cell-laden hydrogel is filled into the microfluidic channel and a vascular network is formed by the encapsulated endothelial cells resulting from spontaneous angiogenesis driven by interstitial perfusion (Phan et al., 2017).

vasculature was formed into the 3D matrix. Astrocytes and neurons could then be introduced to the right side of the hydrogel section and interact physically with the vascular network. This set-up allowed for usage of two different cell culture media, introduced on either side of the hydrogel compartment, each optimised for the specific cell types used. The use of specialized culture medium for the different cells resulted in the formation of a tighter barrier compared to previous developed models. The system used by Bang et al. is rather complicated in design but a single microfluidic channel can also be partitioned into several subchannels by co-flowing hydrogel solutions and liquids s presented by Wong et al. (2008). D.T.T. Phan et al. have also demonstrated the development of a vascularised network form spontaneously by the encapsulated cells. In their work, they tailored the hydrogel's properties to allow for an interstitial flow driven by a hydrostatic pressure system. This slow flow served to guide the endothelial cells to self-organise into a vasculature network (Phan et al., 2017).

Microfluidic systems can also be used for long-term culture of cell-laden constructs. For example, Skardal et al. fabricated 3D liver constructs in a microfluidic device consisting of four circular chambers with a diameter of 10 mm with independent fluidic channels and used it to study the effects of cell toxicity of different concentrations of methanol. A HA/Gelatin/PEG-DA hydrogel solution containing HepG2 liver cells was introduced in each chamber and the hydrogel was defined into 4 mm diameter constructs with a height of 150 μm using UV lithography. The tissue constructs were then cultured for 7 days inside the device under perfusion (Skardal et al., 2015). S. Lee et al. demonstrated a similar method to prepare cell-laden hydrogel constructs defined inside a microfluidic channel by using a modified stereolithography set-up to make it more cell-friendly (Lee et al., 2009a). In their work, they showed the possibility to encapsulate HeLa cells in the PEG-based hydrogel to print multilayer structures in a microfluidic chip for long-term cell culture and cell-based analysis. Recently, a non-contact method for long-term culture of cell-laden hydrogels under perfusion was presented by A. Fornell et al. when hyaluronic acid microgels were introduced into an acoustic trap. This device is compatible with optical microscopy allowing for *in situ* imaging of the cell culture during culture and immunofluorescent staining (Fornell et al., 2019).

Conclusions

This review presents the evolution and the current state-of-the-art of microfabricated hydrogels used for *in vitro* cell culture scaffolds. Different microfabrication techniques are described and important aspects of hydrogel cross-linking have been discussed. We have shown that there are immense possibilities within life science and medical research to prepare *in vitro* models for advanced biological studies using microfabrication techniques. The various techniques described enables chemical, mechanical and topological definition of hydrogel based 3D culture scaffolds. The challenge often lies in combining the right material and technique to ensure good substrate definition with minimum adverse effects on the cells. For example, UV lithography is a versatile tool capable of preparing samples with high resolution (only a few μm's in line width of a chemical pattern) and large scale (to a few mm's sample size) but the cytotoxicity of the cross-linking radicals limit its use for cell-laden scaffolds. Here, 2PP is a good alternative as it is operating in the IR spectra, which is not cytotoxic. 2PP, on the other hand, has a very slow operation speed which may limit its use for high-throughput studies.

We hope that this review article has clearly presented the possibilities and limitations of the different microfabrication techniques to make the choice easier for new researchers endeavouring into this exciting interdisciplinary research direction.

In addition to the choice of fabrication technique one must also consider the chemical base of the biomimetic scaffold itself. In this manuscript, we have presented the three most commonly used cross-linking pathways that can be used to define surface and bulk hydrogel based cell cultures. Interestingly, the spectrum of cross-linking chemistries used today for *in vitro* models is surprisingly small compared with methods used for hydrogel formation in tissue regeneration applications. Therefore, many concepts for non-toxic, bioorthogonal cross-linking chemistries (i.e. chemistries that does not interfere with the native biochemical processes) could be translated from related research fields working with 3D cell culture in hydrogels and we anticipate such a development in the future.

Taken the amount of efforts in combining expertise from microsystems engineering, biology and material science we believe that the number of manuscripts and research programmes combining these techniques will rapidly increase in the future.

Declaration of competing interest

The authors confirm that there are no conflicts to declare.

Acknowledgements

This work is funded by the European research council through an ERC Starting Grant awarded to MT (ERC-2017-STG-757444), The Knut and Alice Wallenberg Foundation through a Wallenberg Fellowship held by MT (KAW.2016.0112), Uppsala University, The Swedish Research Council FORMAS (216-2014-1247) and the National Council of Science and Technology of Mexico through partial funding of the doctoral studies of AMPH. MA is supported by the Marie Curie Fellowship program (grant no. 746270/H2020-MSCA-IF-2016) and the Novo Nordisk Foundation (grant no. NNF16OC0020792). The Novo Nordisk Foundation Center for Stem Cell Research is supported by the Novo Nordisk Foundation (grant no. NNF17CC0027852). We greatly acknowledge the efforts by Sujit Kootala, Uppsala University for careful proof-reading of the section “Hydrogel cross-linking strategies”. Riccardo Cantoni is acknowledged for preparing the schematic images used in the manuscript and Anna Fornell and Zhenhua Liu (both Uppsala University) are thanked for providing the optical images used in Fig. 8.

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